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16. ABSTRACT

A systematic comparison was undertaken to characterize neurotoxic esterase (NTE) from rat and chicken brain in terms of inhibitor sensitivities, pH optima, and molecular weights. Paraoxon titration of phenyl valerate (PV)-hydrolyzing carboxylesterases showed that rat esterases were more sensitive than chicken to paraoxon inhibition at concentrations ≤1 μM and superimposable with chicken esterases at concentrations of 2.5–1000 μM. Mipafox titration of the paraoxon-resistant esterases at a fixed paraoxon concentration of 100 μM (mipafox concentration: 0–1000 μM) resulted in a mipafox I50 of 7.3 μM for chicken brain NTE and 11.6 μM for rat brain NTE. NTE (i.e., paraoxon-resistant, mipafox-sensitive esterase activity) comprised 80% of chicken and 60% of rat brain paraoxon-resistant activity with the specific activity of chicken brain NTE approximately twice that of rat brain NTE. The pH maxima for NTE from both species was similar showing broad, slightly alkaline optima from pH 7.9 to 8.6. [^{3}H]Diisopropyl phosphorofluoridate (DFP)-labeled NTE from the brains of both species had an apparent mol wt of 160,000 measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In conclusion, NTE from both species was very similar, with the mipafox I50 for rat NTE within the range of reported values for chicken and human NTE, and the inhibitor parameters of the chicken NTE assay were applicable for the rat NTE assay.

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An *in Vitro* Comparison of Rat and Chicken Brain Neurotoxic Esterase

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An *in Vitro* Comparison of Rat and Chicken Brain Neurotoxic Esterase. NOVAK, R., AND PADILLA, S. (1986). *Fundam. Appl. Toxicol.* 6, 464–471. A systematic comparison was undertaken to characterize neurotoxic esterase (NTE) from rat and chicken brain in terms of inhibitor sensitivities, pH optima, and molecular weights. Paraoxon titration of phenyl valerate (PV)-hydrolyzing carboxylesterases showed that rat esterases were more sensitive than chicken to paraoxon inhibition at concentrations ≤1 μM and superimposable with chicken esterases at concentrations of 2.5–1000 μM. Mipafox titration of the paraoxon-resistant esterases at a fixed paraoxon concentration of 100 μM (mipafox concentration: 0–1000 μM) resulted in a mipafox *I*50 of 7.3 μM for chicken brain NTE and 11.6 μM for rat brain NTE. NTE (i.e., paraoxon-resistant, mipafox-sensitive esterase activity) comprised 80% of chicken and 60% of rat brain paraoxon-resistant activity with the specific activity of chicken brain NTE approximately twice that of rat brain NTE. The pH maxima for NTE from both species was similar showing broad, slightly alkaline optima from pH 7.9 to 8.6. [^{3}H]Diisopropyl phosphorofluoridate (DFP)-labeled NTE from the brains of both species had an apparent mol wt of 160,000 measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In conclusion, NTE from both species was very similar, with the mipafox *I*50 for rat NTE within the range of reported values for chicken and human NTE, and the inhibitor parameters of the chicken NTE assay were applicable for the rat NTE assay. © 1986 Society of Toxicology.

Organophosphate-induced delayed neuropathy (OPIDN), a distal axonopathy of the larger-diameter and longer fibers of the central and peripheral nervous systems, is known to develop after acute or during chronic exposures to some organophosphorus (OP) esters (Barnes and Denz, 1953; Cavanagh, 1964; Abou-Donia, 1981). Studies of OPIDN in chicken have led to the identification of a putative biochemical lesion: the inhibition and "aging" of a carboxylesterase, termed neurotoxic esterase or neuropathy target enzyme (NTE) (Johnson, 1969a,b, 1970, 1974). Although NTE's causal role in the development of OPIDN has not yet been proven, its role as a biochemical screen for the delayed neurotoxic potential of organophosphorous compounds has been extensively documented in chickens and some farm animals (Johnson, 1975a,b; Lotti and Johnson, 1978; Soliman, 1983). OP compounds that inhibit chicken brain NTE by approximately 65% and are capable of "aging" (i.e., loss of an alkyl group resulting in a permanently inhibited enzyme) present the requisite conditions for the initiation of OPIDN (Johnson, 1970, 1975a; Clothier and Johnson, 1980; Williams, 1983). The *in vitro* measurement of NTE activity employs a synthetic substrate, phenyl valerate (PV) and separates NTE activity from other esterase activity by its differential response to two organophosphate inhibitors: paraoxon (*O,O,*-diethyl *O*,4-nitrophenylphosphate), a nonneurotoxic OP compound which inhibits the nonspecific esterases; and mipafox (*N,N'*-diisopropylphosphorodiamidic fluoride), a neurotoxic OP compound which inhibits NTE (Johnson, 1982).

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The chicken is traditionally used as a model for OPIDN because rodents are less susceptible to the ataxia characteristic of OPIDN, possibly because their NTE is less sensitive to inhibition by neurotoxic OP compounds (Johnson, 1975c; Hussain and Oloffs, 1979; Soliman *et al.*, 1982; Soliman, 1983). However, recent studies by Veronesi (1984a,b) have demonstrated that the rat does develop a neuropathy that is topographically and qualitatively similar to other animal models of OPIDN in the absence of ataxia, and the brain and spinal cord NTE inhibition correlates with the incidence of neuropathic response in tri-*o*-cresyl phosphate-treated rats (Padilla and Veronesi, 1985). In the latter study it was noted that although the percentage of NTE inhibition predictive of OPIDN in the rat and chicken were similar, there were differences between rat and chicken brain with respect to the time course of NTE inhibition and recovery, again possibly because of differences between the two species in NTE sensitivity to inhibitors. As yet, there is a paucity of investigations systematically comparing NTE from rat and chicken. Ideally, comparison of inhibitor sensitivities should include traditional enzyme kinetic determinations, but these studies cannot be begun until NTE has been isolated, although some enriched fractions have been obtained (Ishikawa *et al.*, 1983; Chemnitius *et al.*, 1984).

The present study was undertaken to determine *in vitro* the sensitivity of rat and chicken brain PV-hydrolyzing esterases to paraoxon, and to determine the mipafox *I*50 values of their respective NTEs. In addition, pH optima and apparent molecular weights of chicken and rat brain NTE were determined.

MATERIALS AND METHODS

Chemicals

Paraoxon (≥97% pure) was obtained from Aldrich Chemical Company (Milwaukee, Wisc.). Mipafox and phenyl valerate were generously supplied by Dr. S. Soliman (Alexandria, Egypt). [1,3-^{3}H]Diisopropyl phosphorofluoridate (DFP: 5.2 Ci/mmol) and Protosol were obtained from New England Nuclear, Inc. (Boston, Mass.), molecular weight standards from Bio-Rad (Richmond, Calif.), and all other chemicals from Sigma Chemical Company (St. Louis, Mo.) or Fisher Scientific Company (Pittsburgh, Pa.).

Animals and Tissue Preparation

Long–Evans hooded male rats, 60–90 days old (Charles River, Inc., Wilmington, Del.) and adult white leghorn chickens, 1–1.5 years old (Featherdown Farm, Apex, N.C.), were used. Animals were sacrificed by decapitation, brains were removed, weighed, and immediately placed in ice-cold buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0). Chicken brains were homogenized in 7 vol and rat brains in 5 vol of buffer for 15–20 sec at medium speed with a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y.). The whole brain homogenates were centrifuged at 9000*g* for 15 min, 4°C (Sorvall RC-5). These supernatants were used the same day for enzyme analysis.

NTE Assay

The NTE assay method of Johnson (1977) as modified by Soliman *et al.* (1982) was used with additional variations as noted. Duplicate tissue samples containing 0.8–0.9 mg Lowry protein were incubated for 20 min at 37°C in paired sets of tubes containing buffer (50 mM Tris, 0.2 mM EDTA, pH 8.0) with either (A) paraoxon or (B) paraoxon plus mipafox (final vol, 3.0 ml). After the preincubation, PV (0.93 mM) was added to start the enzymatic reaction. After 10 min (both rat and chicken brain assays were linear over this time interval) the reaction was stopped by the addition of 1.0% sodium dodecyl sulfate (SDS) with 0.025% 4-amino antipyrine (dissolved in Tris buffer, pH 8). The product was reacted with 0.4% $K_3Fe(CN)_6$ to give a stable red color. Absorbance was measured at 510 nm on a Guilford 260 spectrophotometer and NTE activity calculated as the difference in absorbance between tubes A and B. Total PV-hydrolyzing esterase activity was assayed as above but in the absence of inhibitors. Unless otherwise indicated, assay concentration of paraoxon was 40 μM and that of mipafox was 50 μM.

Titration of Inhibitors

Assays were carried out as described above with the following modifications.

(1). Paraoxon titration. Each brain sample was added to duplicate tubes containing paraoxon at 16 different concentrations ranging from 0.01 to 1000 μM. Paired tubes containing no paraoxon were used to determine the total

PV-hydrolyzing esterase activity of the sample (i.e., 100% activity).

(2). Mipafox titration. Each brain sample was added to duplicate tubes containing paraoxon (100 μM) and mipafox at 15 different concentrations (0.01 to 1000 μM). Paired tubes containing 100 μM paraoxon and no mipafox were included with each assay to measure total paraoxon-resistant esterase activity (i.e., 100% activity).

Calculation of Mipafox I50 Values

To determine the *I*50 values for mipafox, a four-parameter logistic function (see legend, Table 1) was used to model the NTE inhibition curve (DeLean *et al.*, 1978). The NTE activity data were fit to this function by the procedure NLIN of SAS (SAS Institute, Inc., 1982), an iterative technique that fits the model by least squares.

pH Effect

When assaying for NTE activity, citrate buffer from pH 5.0 to 6.5 and Tris buffer from pH 7.0 to 9.0 produced a discontinuous response in both rat and chicken brain tissue. Therefore a single buffer system (100 mM Tris–HCl/base, 0.2 mM EDTA) ranging from pH 5.9 to 8.6 was used to determine pH optima.

Radiolabeling of NTE

Chicken and rat brain microsomes were labeled with [^{3}H]DFP according to the method of Williams and Johnson (1981). In brief, this method involves the same differential inhibition principles used in the NTE assay. Microsomes were preincubated with either paraoxon or paraoxon plus mipafox, separated from unbound inhibitors by centrifugation, and then incubated with [^{3}H]DFP which binds to NTE. Theoretically, NTE is available for DFP binding only in the microsomes preinhibited with paraoxon and not in those preinhibited with paraoxon plus mipafox, since mipafox should have already bound to the esteratic site of NTE. Labeled microsomes were separated from free radiolabel by centrifugation and were then lyophilized for subsequent gel electrophoresis.

Electrophoresis

Tubular gel (6 × 120 mm) electrophoresis was carried out on 7.5 and 10.0% polyacrylamide gels with a 5% stacking gel (Laemmli, 1970; Maizel, 1971). Equal amounts of protein (Lowry *et al.*, 1951) were electrophoretically separated at 100 V until the dye front reached the bottom of the gel. One of each group of tubular gels contained the following molecular weight standards: phosphorylase B (94,000), bovine serum albumin (66,000), and ovalbumin (43,000), while another gel contained delipidated rat brain cytoskeleton proteins, isolated according to the method of Chiu and Norton (1982). Molecular weight estimation was performed according to Lambin (1978). After electrophoresis, the gels were fixed, stained, destained, and sectioned into 1.1-mm slices for radioactivity determination (Padilla and Morell, 1980). Scintillation counting was done on a Beckman LS 7000 scintillation counter. Counting efficiency for the ^{3}H channel was approximately 26%.

RESULTS

Inhibitor Titration

Paraoxon titration curves for inhibition of rat and chicken brain PV-hydrolyzing activity closely resembled each other and were not significantly different at paraoxon concentrations of 2.5 μM or greater (Fig. 1).

The mipafox titration curves for the inhibition of rat and chicken brain paraoxon-resistant esterase activity were sigmoidal between 1.0 and 75 μM mipafox in both rat and chicken brain (Fig. 2) and the data fit well to the model curve (DeLean *et al.*, 1978). The equation parameters, calculated I50 values, and 95% confidence limits for the inhibition of NTE activity in both chicken and rat brain are presented in Table 1. Mipafox *I*50 values were calculated to be 11.6 μM for rat brain NTE and 7.3 μM

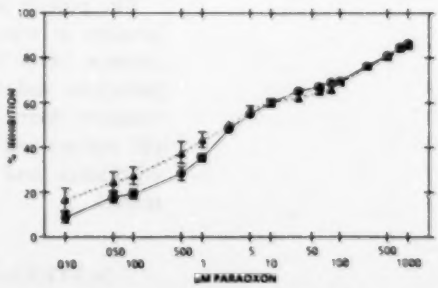

FIG. 1. Titration of PV-hydrolyzing activity in rat (▲) and chicken (●) brain with increasing concentrations of paraoxon. Each curve (mean ± SE of three brains) is expressed as a percentage of the total PV-hydrolyzing activity in each sample. 100% esterase activity in rat = 59.6 ± 1.3; in chicken = 107.9 ± 2.6 nmol phenyl valerate hydrolyzed/min/mg protein.

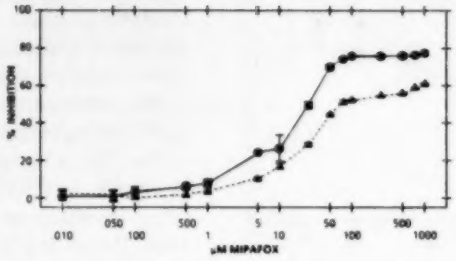

FIG. 2. Titration of the paraoxon-resistant PV-hydrolyzing activity in rat (▲) and chicken (●) brain in the presence of increasing concentrations of mipafox. All assays were done in the presence of 100 μM paraoxon. Each curve represents the mean ± SE of three brains from each species. 100% paraoxon-resistant activity in rat = 19.6 ± 0.51; in chicken = 33.4 ± 0.51 nmol phenyl valerate hydrolyzed/min/mg protein.

for chicken brain NTE. Above concentrations of 75 μM mipafox in both curves, the PV-esterase activity showed little further inhibition across a 10-fold increase in mipafox concentration. At these concentrations of mipafox, the chicken brain showed a higher degree of PV-esterase inhibition (80%) than did the rat brain (60%).

pH Effect

NTE activity for both species over the pH range of 5.9 to 8.6 did not result in a well-defined pH optimum (Fig. 3). Rat and chicken brain NTE activity increased by only 40 and 36%, respectively, from pH 5.9 to 7.9 with no additional increase up to pH 8.6. The specific activity of chicken brain NTE under normal assay conditions was approximately twice that of rat brain (see legend, Fig. 3).

[^{3}H]DFP Labeling

Microsomal proteins, radiolabeled with [^{3}H]DFP after preincubation with paraoxon and separated on SDS polyacrylamide gels produced a single peak of [^{3}H] activity with an apparent mol wt of 160,000 in both chicken and rat brain (solid lines, Fig. 4). Almost complete supression of the [^{3}H]DFP labeling of this peak was accomplished by the addition of mipafox to the preincubation mixture (broken lines, Fig. 4). Although equal amounts of Lowry proteins were applied to each gel, the area of the labeled NTE peak from chicken brain is approximately twice that of the labeled NTE peak from rat brain.

DISCUSSION

While the chicken brain NTE specific activity was two times that of rat brain, the present data show that rat and chicken brain NTE responded in a qualitatively similar fashion when subjected to the same variables. Small, species-specific quantitative differences were noted with respect to the PV esterase sensitivity to inhibition by paraoxon and mipafox. Rat brain PV hydrolysis was slightly more sensitive to paraoxon inhibition at concentrations of 0.01 to 1 μM, which represents inhi-

TABLE 1[a]

	Rat	Chicken
d = Minimum activity[b]	7.82 (7.55, 8.12)[c]	7.55 (6.99, 8.12)
a = Maximum activity[b]	19.38 (19.12, 19.64)	32.37 (31.73, 33.00)
b = Slope	1.13 (1.00, 1.25)	1.59 (1.32, 1.86)
c = I50 value (μM)	11.6 (10.2, 12.9)	7.3 (6.5, 8.1)

[a] The general form of the symmetrical, sigmoidal curve is given by the equation (DeLean *et al.*, 1978) $Y = (a - d)/[1 + (x/c)^b] + d$.

[b] Activity is expressed as nmol phenyl valerate hydrolyzed/min/mg protein.

[c] Numbers in parentheses represent the approximate 95% confidence limits.

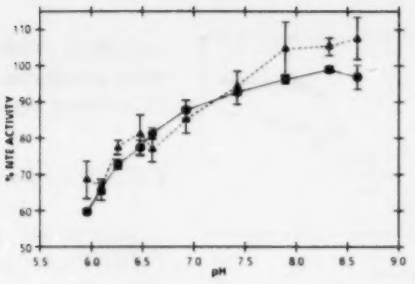

FIG. 3. pH sensitivity of NTE from brains of both rat (▲) and chicken (●). The pH of the final reaction mixtures at 37°C is indicated on the abscissa. All activity is expressed as percentage of the NTE activity under normal assay conditions which were 11.4 ± 0.4 in the rat and 26.0 ± 1.6 nmol phenyl valerate hydrolyzed/min/mg protein in the chicken.

bition of paraoxon-sensitive esterases (Johnson, 1969b; Davis and Richardson, 1980). At the higher paraoxon concentrations (25–100 μM), where the degree of enzyme inhibition in both species was identical, all paraoxon-sensitive esterases were inhibited and yet the paraoxon-resistant esterase activity was retained (Johnson, 1969b, 1975c). For this reason a paraoxon concentration of 40 μM for the chicken NTE assay has been used (Johnson, 1975c, 1977). Because rat and chicken brain paraoxon titration curves were superimposable at this point, a 40 μM concentration of paraoxon would also be applicable for the rat NTE assay.

For the mipafox titration curves a constant concentration of 100 μM paraoxon was chosen in order to assure complete elimination of paraoxon-sensitive esterase activity. Paraoxon-resistant esterase activity is comprised of two types of activity (Johnson, 1969b; 1982): one type is sensitive to mipafox and represents NTE activity (Johnson, 1969b), while the remaining type of esterase activity is referred to as mipafox-resistant (Olajos *et al.*, 1979) or inhibitor-resistant esterase activity (Richardson *et al.*, 1979). As shown in our mipafox titration curves, the portion of paraoxon-resistant esterase activity representing NTE activity was inhibited by 1.0 to 75 μM mipafox while at concentrations greater than 75 μM the inhibitor-resistant esterases continued to withstand inhibition by mipafox. The difference in inhibition between the rat and chicken in this latter portion of the mipafox titration curve indicates that approximately 70–80% of chicken brain paraoxon-resistant esterases can be classified as NTE, as has been reported by other investigators (Davis and Richardson, 1980; Dudek and Richardson, 1982; Williams, 1983; Olajos *et al.*, 1979). The rat appeared to have less NTE activity (60%) as a percentage of its total paraoxon-resistant esterase activity. Note that the concentration range over which NTE activity could be detected was identical in both chicken and rat, an indication that the 50 μM mipafox concentration traditionally used in chicken NTE assays inhibited the majority of brain NTE activity in both species.

An *in vitro* mipafox *I*50 value of 7.3 μM in chicken brain was within the range of values (7–10 μM) reported by other investigators (Lotti and Johnson 1978; Caroldi and Lotti,

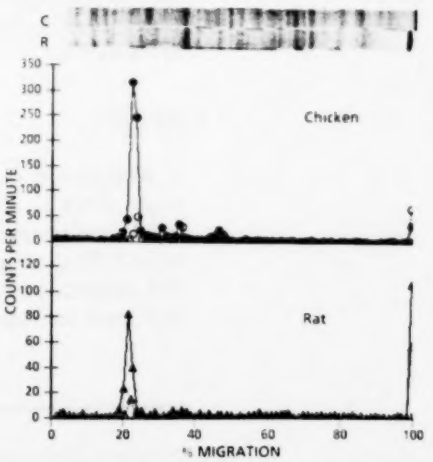

FIG. 4. Representative gels of the electrophoretic separation of [^{3}H]DFP-labeled microsomal proteins from chicken (C) and rat (R) brain above the radioactive profile of the sliced gels. Solid lines represent labeling in presence of 160 μM paraoxon while dotted lines represent labeling in the presence of 160 μM paraoxon and 50 μM mipafox. Equal amounts (250 μg) of lowry protein were applied to each gel.

1982; Ishikawa *et al.*, 1983; Williams, 1983). The mipafox *I*50 value of 11.6 μM for rat brain NTE was outside the approximate confidence limits of our calculated *I*50 value for chicken brain NTE. Nevertheless, it is interesting to note that the mipafox *I*50 value for rat brain NTE is nearer the mipafox *I*50 of 12–14 μM calculated from human tissues by Lotti and Johnson (1978, 1980). These results suggest that rat brain NTE, rather than chicken brain NTE, may more closely resemble human NTE with respect to *in vitro* neurotoxic OP inhibitor sensitivity.

Both rat and chicken brain NTE responded to variations in buffer pH in the same manner and with little evidence of a pH optimum for NTE activity in the range investigated (pH 5.9 to 8.6). This broad, slightly alkaline pH optimum has been reported for chicken brain NTE (Johnson, 1982), is common to many carboxylesterases (Junge and Krisch, 1975), and may be due to the use of a nonphysiological substrate.

This study also demonstrated that both rat and chicken brain microsomes, preincubated with paraoxon and labeled with [^{3}H]DFP, produced peaks of equivalent molecular weight as determined by polyacrylamide gel electrophoresis. In addition, the presence of the neurotoxic organophosphate, mipafox, inhibited the labeling of this microsomal protein. This mipafox-sensitive, 160,000 molecular weight protein in both rat and chicken brain represented a molecular weight similar to other reports for chicken brain NTE (Williams and Johnson, 1981; Williams, 1983), or in general, to many liver carboxylesterases (Junge and Krisch, 1975). This study demonstrates that the specific activity of chicken brain NTE was approximately two times higher than that in rat brain (see legend, Fig. 3) which correlates well with the observation that the chicken brain microsomal 160,000 molecular weight protein (NTE) showed twice the ^{3}H labeling with DFP than did the rat brain protein.

However, NTE represents only one facet of the cumulative, delayed neurotoxic response. Inter- and intraspecies differences in sensitivity to OPIDN are well known and have been postulated to be due to diversity in OP absorption and distribution (Abou-Donia, 1983), bioactivation (Hansen, 1983; Veronesi, 1984b), nervous system structure (Johnson, 1975c), or neuronal repair mechanisms (Richardson, 1984). Of the above variables, pharmacokinetics and metabolism of OP compounds are accepted as playing prominent roles in OP susceptibility (for an overview see Abou-Donia, 1983; Hansen, 1983). The distinction between susceptible and nonsusceptible animals may not be centered in the nervous system per se, rather the "insensitive" animals may detoxify and excrete the OP compound more rapidly and completely than the "sensitive" animals. Examples of this species-specific difference have been noted in chickens and rats treated with *O*-4-bromo-2,5-dichlorophenyl-*O*-methyl phenylphosphonothioate (i.e., leptophos; Hassan *et al.*, 1977; Abou-Donia and Ashry, 1978; Abou-Donia, 1980) or *O*-ethyl-*O*-(4-nitrophenyl) phenylphosphonothioate (i.e., EPN; Chrzanowski and Jelinek, 1981). Even within a species, genetic differences (Lee, 1964) as well as pharmacological manipulations (Calabrese and Bursian, 1984; Veronesi, 1984b) influence the delayed neurotoxic response, presumably through biochemical changes which control the duration, extent, distribution, and metabolite identification of the OP body burden. Therefore, similarities regarding *in vitro* sensitivity to OP compounds may not necessarily reflect *in vivo* similarities in the neurotoxic response.

In summary, the data presented here establish the biochemical similarities between chicken and rat brain NTE on the basis of their response to inhibitor concentrations and change in pH, and their characterization by radiolabeling and gel electrophoresis. This *in vitro* comparison of rat and chicken brain NTE provides further validation of the rodent model of OPIDN and, coupled with the knowledge that the rat develops OP-induced neuropathic lesions, should encourage research using this more familiar mammalian

species as an experimental model for addressing the relationship between NTE and the neuropathic response.

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